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Serum levels of matrix metalloproteinase-2 and -9 and conventional tumor markers (CEA and CA 19-9) in patients with colorectal and gastric cancers

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Abstract

Background: Matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, play an important role in tumor invasion and metastasis. This study aimed to determine the serum levels of MMP-2, MMP-9, 130- and 225-kDa gelatinolytic bands and conventional tumor markers, carcinoembryonic antigen (CEA) and cancer antigen (CA) 19-9, in patients with gastrointestinal cancers. The relationship between these parameters and clinicopathological factors was also studied.
Methods: Sera from controls (n=19), and patients with colorectal (n=47) and gastric (n=34) cancer were collected prospectively. The gelatinolytic activities of MMP-2, MMP-9, 130- and 225-kDa bands were determined using gelatin zymography. CEA and CA 19-9 were determined using immunoradiometric assay (IRMA).
Results: Serum levels of MMP-9, 130- and 225-kDa gelatinolytic bands, CEA, and CA 19-9, but not MMP-2, in colorectal and gastric cancer were significantly higher than that of controls. No significant correlation was found between histological grade or clinical stage and levels of MMP-9, 130- and 225-kDa gelatinolytic bands, which were correlated ($r=0.61–0.89$, $p<0.005$).
Conclusions: Our findings suggest that zymographic determination of MMP-9, 130- and 225-kDa gelatinolytic bands in colorectal and gastric cancer may be useful in studying these types of cancer in parallel with conventional tumor markers.
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Keywords: cancer antigen 19-9 (CA 19-9); carcinoembryonic antigen (CEA); colorectal cancer; gastric cancer; matrix metalloproteinases (MMPs).

Introduction

Remodeling of normal and diseased tissue, whose etiology includes matrix degradation or fibrosis, are thought to result from alterations in the balance between matrix metalloproteinases (MMPs) and tissue inhibitor metalloproteinases (TIMPs) (1). The first barrier for an invading epithelial tumor is the basement membrane, consisting primarily of type IV collagen. The type IV collagenases, matrix metalloproteinase-2 (MMP-2) and MMP-9 (constitutive and inducible gelatinase, respectively), degrade type IV and V collagen, gelatin, and fibronectin (2).

The role of MMP-2 and MMP-9 has been confirmed in colorectal cancer (3–5) and gastric cancer (6–8). Increased levels of MMP-2 and MMP-9 in patients with colorectal and gastric cancer were detected in serum (9, 10), plasma (3, 9), and tissue (3, 11, 12). Different techniques were utilized for the determination of MMP-2 and MMP-9 including gelatin zymography (11, 13), enzyme linked immunosorbant assay (ELISA) (3, 14), immunohistochemical techniques (15), in situ hybridization (2) and reverse transcriptase-polymerase chain reaction (3).

In addition to MMP-2 and MMP-9, the 130- and 225-kDa gelatinolytic bands were detected in zymographic analysis of serum samples and found to be increased in different types of cancer (12, 16). It was confirmed that these bands are MMPs (17). The 225-kDa band corresponded to pro-MMP-9 dimers, while the 130-kDa band had the same molecular size as the MMP-9-lipocalin-2 complex found primarily in neutrophils and other cell types (18).

In this study, gelatin zymography was used to measure concentrations of MMP-2 (72-kDa), MMP-9 (92-kDa), 130- and 225-kDa moieties. We examined the relationship between the concentration and progression of colorectal and gastric cancers as evidenced by either histological grade or clinical stage, and compared this to conventional tumor markers including carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA 19-9).

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Materials and methods

Patients

This prospective study was approved by the Ethics Committee of Wroclaw University of Medicine, Wroclaw, Poland. The study included 47 patients with colorectal cancer, 34 patients with untreated gastric cancer, and 19 healthy volunteers as normal controls. Colorectal and gastric cancer was classified according to the histological grade (G) and clinical stage (S). Histological grading was assessed according to Sternberg (19). Clinical staging was assessed according to Union Internacional Contra la Cancrum (NICC) tumor node metastasis (TNM) classification, where post-surgical histopathological classification (*p*TNM) was used (20).

Gelatin zymographic analysis

The gelatinolytic activity present in serum was determined using gelatin zymographic analysis as described by Kleiner and Stetler-Stevenson (21) with minor modifications. Serum samples were diluted (1:75) in formulated buffer (5 mM Tris-HCl; Sigma, St. Louis, MO, USA), 0.1 mM $CaCl_2$ (Sigma), and 1 mM 1,10-phenathroline (Sigma), pH 7.5) mixed with 4x sample buffer. Samples were electrophoresed in duplicate (20 μL/lane) on 7.5% SDS-PAGE (0.75 mm thickness) containing gelatin (Sigma) (2 mg/mL) using mini-PROTEAN II system (Bio-Rad, Hercules, CA, USA). The concentrated supernatant of the cell culture media (HT-1080 cell line) was used as positive controls for MMP-2 and -9. Following electrophoresis, gels were washed 3 times (20 min each) in 2.5% Triton-X 100 (Sigma-Aldrich, Steinheim, Germany), and then incubated in Tris buffer [0.05 M Tris-HCl, containing 0.01 M $CaCl_2$, 0.2 M NaCl (Merck, Darmstadt, Germany)] and 0.05% NaN_3 (Sigma), pH 7.5 at 37°C for 18 h. Gels were stained with 0.5% Coomassie brilliant blue R250 (Sigma) in 30% methanol (POCh, Poland), and 10% acetic acid (POCh) (100 mL/gel) for 3 h with shaking at room temperature, and then destained with three changes of 30% methanol, and 10% acetic acid (100 mL/gel) for 15, 30, and 60 min of destain time, respectively. Gels were scanned and analyzed with SigmaGel software (Jandel Corporation, San Rafael, CA, USA), where the integrated area of each gelatinolytic band was measured. The size of the gelatinolytic bands were expressed in arbitrary units (AU).

Determination of tumor markers in human sera

CEA and CA 19-9 were measured in human sera, using CEA-IRMA (immunoradiometric assay) (Radioisotope center, Polatom, Warsaw, Poland) and CA 19-9 IRMA (CIS Bio International, Gif-sur-Yvette Cedex, France) kits, respectively.

Statistics

Data are expressed as mean ± SEM. Non-parametric analysis [Kruskal-Wallis analysis of variance (ANOVA)] was used to compare differences between groups. Post hoc analysis by Dunn's test was performed when the $p < 0.05$. Correlations between the two groups were analyzed using Pearson moment or Spearman R test for parametric and non-parametric variables, respectively. Statistical analysis was performed using statistical software (GraphPad Software, Inc., San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

Results

Patient demographics

Patients with colorectal (n = 47, 64 ± 2 years) and gastric (n = 34, 64 ± 2 years) cancer were grouped according to their histological grade and clinical stage. Histological grading showed that the number of patients with colorectal cancer GI, GII and GIII were 5, 38 and 4, respectively. Histological grades of patients with gastric cancer GI, GII and GIII were 2, 16 and 16, respectively. Clinical staging revealed that the number of patients with colorectal cancer S0, SI, SII, SIII and SIV were 0, 4, 16, 11 and 16, respectively. Staging of patients with gastric cancer SIa, SIb, SII, SIIIa, SIIIb and SIV were 0, 0, 6, 0, 10 and 18, respectively (Table 1).

Gelatin zymographic analysis

Zymographic analysis of serum revealed four gelatinolytic bands corresponding to MMP-2 (72-kDa), MMP-9 (92-kDa), 130- and, 225-kDa gelatinolytic bands. In normal controls and patients with colorectal and gastric cancer, there were no significant differences in the gelatinolytic activities between females and males.

Serum levels of MMP-2

Levels of serum MMP-2 in patients with colorectal (426.5 ± 35.8 AU) and gastric (386.1 ± 26.3 AU) cancer were not significantly higher than in normal controls (319.7 ± 28.3 AU) (Figure 1A). No significant differences were found between histological grade or clinical stage in patients with colorectal or gastric cancer.

Serum levels of MMP-9

Serum levels of MMP-9 in patients with colorectal (1324 ± 85 AU) or gastric cancer (1234 ± 77 AU) were significantly higher than values seen in normal controls (960 ± 79 AU) (Figure 1B). For patients with colorectal cancer, differences in MMP-9 levels between histological grade or clinical stage and normal controls were not significant (Figure 2A and B). However, in patients with gastric cancer, significant differences were found in MMP-9 levels in GI and SIII cancer when compared to normal controls (Figure 2C and D).

Serum levels of 130-kDa and 225-kDa gelatinolytic bands

Serum levels of 130-kDa gelatinolytic band in patients with colorectal (259 ± 29 AU) or gastric (250 ± 27 AU) cancer were significantly higher than in normal controls (116 ± 24 AU) (Figure 1C). Significant differences were found in the levels of 130-kDa gelatinolytic band of GI, GII, SIIIb, and SIV in gastric cancer patients (Figure 2C and D) compared to normal controls. In addition, for patients with colorectal cancer, significant differences were found in the level of 130-kDa gelatinolytic band of GII and SII cancer compared to normal

Table 1 Demographic data of normal controls and patients with colorectal and gastric cancer.

	Control n=19	Colorectal cancer n=47	Gastric cancer n=34
Mean age, years	54±2	64±2	64±2
Female/male	8/11	20/27	10/24
Histological grade			
Grade I			
Mean age, years	–	68±4	54±1
Female/male		3/2	1/1
Grade II			
Mean age, years	–	65±2	64±3
Female/male		14/24	5/11
Grade III			
Mean age, years	–	53±5	66±3
Female/male		3/1	4/12
Clinical stage			
Stage 0			
Mean age, years	–	#	–
Female/male			
Stage Ia			
Mean age, years	–	–	#
Female/male			
Stage Ib			
Mean age, years	–	–	#
Female/male			
Stage I			
Mean age, years	–	63±6	–
Female/male		2/2	
Stage II			
Mean age, years	–	64±3	65±4
Female/male		6/10	2/4
Stage IIIa			
Mean age, years	–	–	#
Female/male			
Stage IIIb			
Mean age, years	–	–	64±4
Female/male			2/8
Stage III			
Mean age, years	–	65±4	–
Female/male		6/5	
Stage IV			
Mean age, years	–	65±2	63±4
Female/male		6/10	6/12

–, not applicable; #, no patients in this category.

controls. A modest increase was seen in patients with GIII, SIII, and SIV cancer (Figure 2A and B).

Serum levels of 225-kDa gelatinolytic band in patients with colorectal cancer (284±24 AU) or gastric cancer (285±20 AU) were significantly higher than those in normal controls (172±18 AU) (Figure 1D). There were significant differences in the levels of 225-kDa gelatinolytic band in GII, GIII, SIIIb, and SIV of gastric cancer (Figure 2C and D) and in GII of colorectal cancer compared to that of normal controls (Figure 2A). In patients with colorectal cancer, there were no significant differences in 225-kDa gelatinolytic band levels with respect to clinical stages and normal controls (Figure 2B).

Serum concentrations of CEA and CA 19-9

There were no significant differences in CEA and CA 19-9 between females and males comprising the control group or patients with colorectal, or gastric cancer. In patients with colorectal cancer, serum concentrations of CEA (19.3±5.9 ng/mL) were significantly higher than in normal controls (0.7±0.2 ng/mL) ($p<0.001$). The serum CEA concentrations with respect to histological grade and clinical stage were significantly different from that of normal controls ($p<0.01$ for both) (Figure 3A and B).

For patients with gastric cancer, serum concentrations of CEA (13.9±4.8 ng/mL) were significantly higher compared with normal controls ($p<0.05$). The serum concentration of CEA in patients with SIV cancer was higher than values seen in normal controls (Figure 3B). There was no significant difference with respect to histological grade and normal control (Figure 3A).

For patients with colorectal cancer (29.4±11.9 U/mL), serum concentrations of CA 19-9 were modestly higher than values seen in normal controls (7.8±1.3 U/mL), but the difference was not significant. Significant difference was found in the concentrations of CA 19-9 in patients with SIV cancer compared to normal controls (Figure 3D). Serum CA 19-9 concentrations

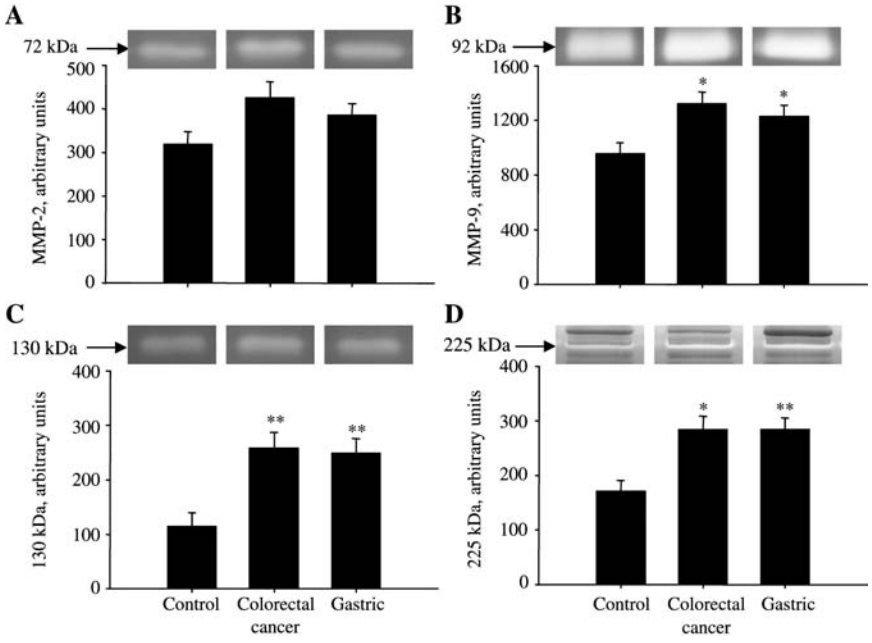

Figure 1 Representative zymograms and levels of MMP-2 (A), MMP-9 (B), 130-kDa (C) and 225-kDa (D) gelatinolytic bands in normal controls, and patients with colorectal and gastric cancer.
**$p < 0.001$ and *$p < 0.05$ vs. control (ANOVA).

with respect to histological grade and normal controls were not significantly different (Figure 3C).

In patients with gastric cancer, serum concentrations of CA 19-9 (71.4 ± 20.7 U/mL) were significantly higher than values seen in normal controls (7.8 ± 1.3 U/mL) ($p < 0.01$). Significant differences were found in the concentrations of CA 19-9 in patients with GII and SIV cancer compared to those of normal controls (Figure 3C and D).

Correlations between measured serum parameters

Significant and positive correlation was found between MMP-9 and 130-kDa MMP-9 and 225-kDa

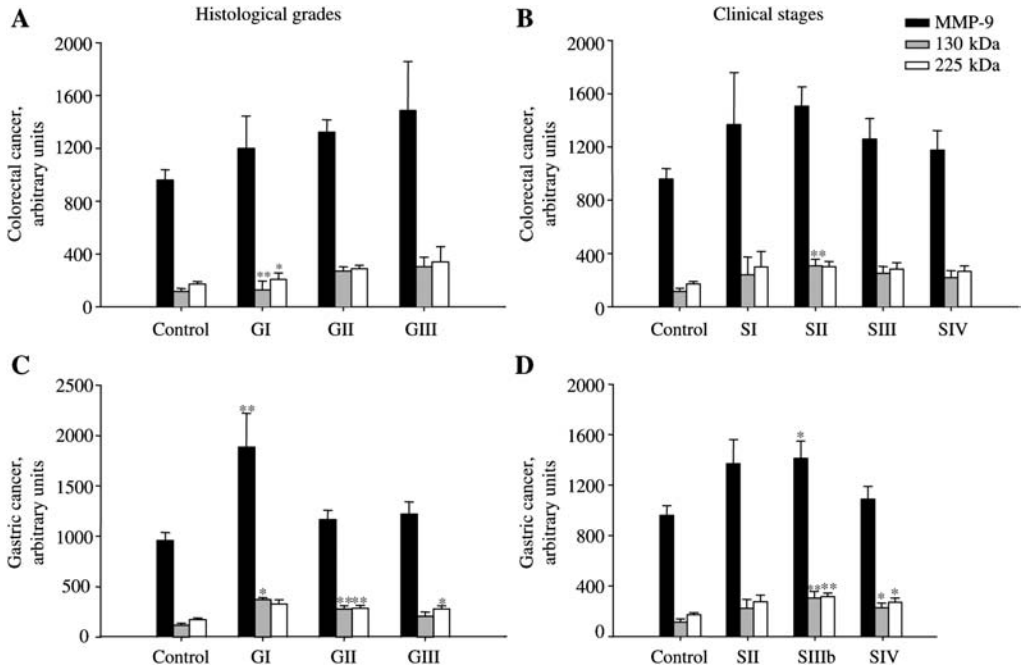

Figure 2 Levels of MMP-9, 130-kDa, and 225-kDa gelatinolytic bands in the sera of patients with colorectal (n = 47; A and B) and gastric (n = 34; C and D) cancer in relation to histological grades (GI–GIII) (left panel) and clinical stage (SI–SIV) (right panel).
**$p < 0.001$ and *$p < 0.05$ vs. control (ANOVA).

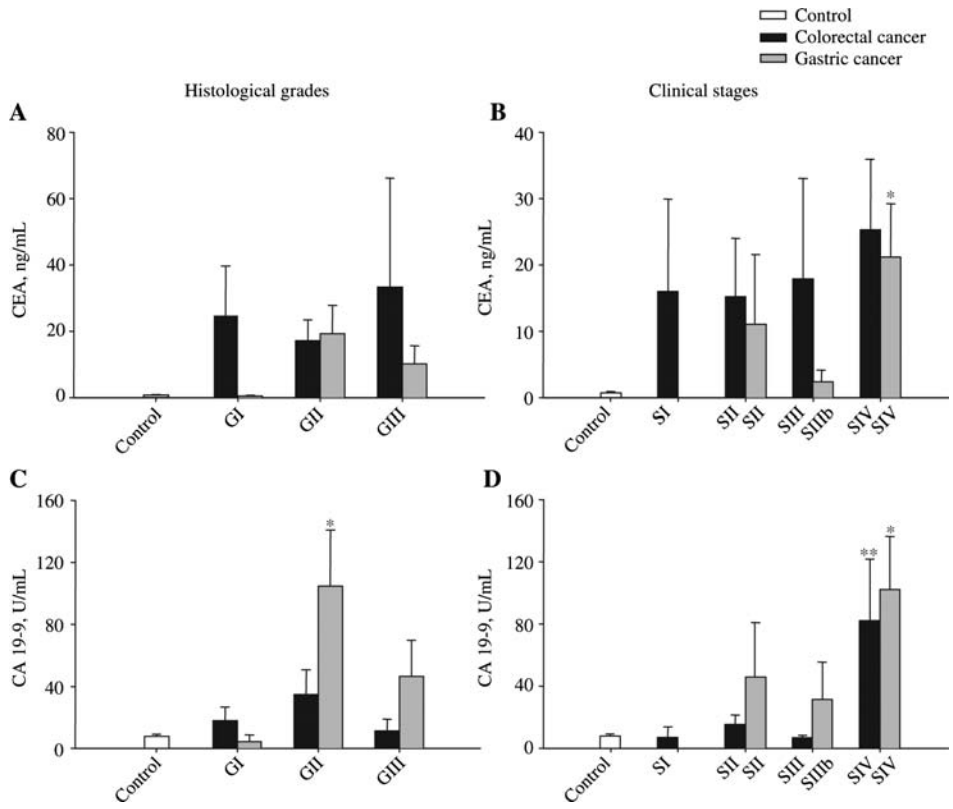

Figure 3 Serum levels of CEA (A and B) and CA 19-9 (C and D) in colorectal and gastric cancer in relation to histological grade (GI–GIII) (left panel) and clinical stage (SI–SIV) (right panel).
**$p < 0.001$ and *$p < 0.05$ vs. control (ANOVA).

values in normal controls ($r = 0.85$, $p < 0.0001$; $r = 0.66$, $p = 0.002$, respectively), patients with colorectal ($r = 0.85$, $p < 0.0001$; $r = 0.89$, $p < 0.0001$) and gastric ($r = 0.71$, $p < 0.0001$; $r = 0.61$, $p < 0.0001$) cancer (Figure 4). No significant correlation was seen between MMP-2 and 130-kDa, MMP-2 and 225-kDa levels in normal controls and patients with colorectal or gastric cancer.

Discussion

MMP-2 and -9 are the most extensively studied MMPs in various types of cancer. However, there is little information about the actual role of 130- and 225-kDa gelatinolytic bands. In this study, the significant increase in serum levels of MMP-9 in patients with colorectal or gastric cancer is consistent with other studies using either gelatin zymography or ELISA to measure MMP-9 in serum (10), plasma (3) and paired samples of colorectal cancer and normal colon mucosa (3, 13). However, there was no correlation between MMP-9 levels and histological grade or clinical stage of the tumor. Despite large variations in samples, Baker and Leaper (11) found that levels of MMP-9 correlated with the Dukes staging and lymphatic invasion of colorectal cancer ($n = 77$). In addition, immunohistochemical studies showed that either serum or tissue MMP-9 expression was significantly correlated with the stage of gastric cancer ($n = 256$) (8, 22, 23). However, others reported no significant correlation between the grade or stage of cancer ($n = 53$–192) and the level of MMP-9 (2, 9, 10, 13) which is similar to our observations. Although the relation between serum MMP-9 levels and histological grade or clinical stage is controversial, we believe that it is partly related to differences in methods used by different studies. The significant increase in serum levels of MMP-9 in patients with colorectal and gastric cancers implies that MMP-9 may be associated with tumor progression.

It has been shown that the 130-kDa gelatinolytic band is a complex of MMP-9 and neutrophil-derived lipocalin-2 (NGAL), and the 225-kDa gelatinolytic band corresponds to pro-MMP-9 dimers (24, 25). The significant and positive correlations found between MMP-9 levels and 130-kDa or 225-kDa gelatinolytic bands in normal controls, patients with colorectal and gastric cancer support 130-kDa and 225-kDa as constituting the heterodimer and homodimer of MMP-9, respectively (25). Also, the significant increase in the 130- and 225-kDa gelatinolytic bands in colorectal or gastric cancer is in accordance with other reports (12, 16). This may reflect their biological abundance and importance in the transformation of a tumor from the benign to the malignant state, and may also indicate their role in tumor progression. It is known that MMP-9, when complexed to Lipocalin-2, is protected from full inhibition by TIMP-1 and retains the capacity to function as an auto-activator for non-complexed MMP-9

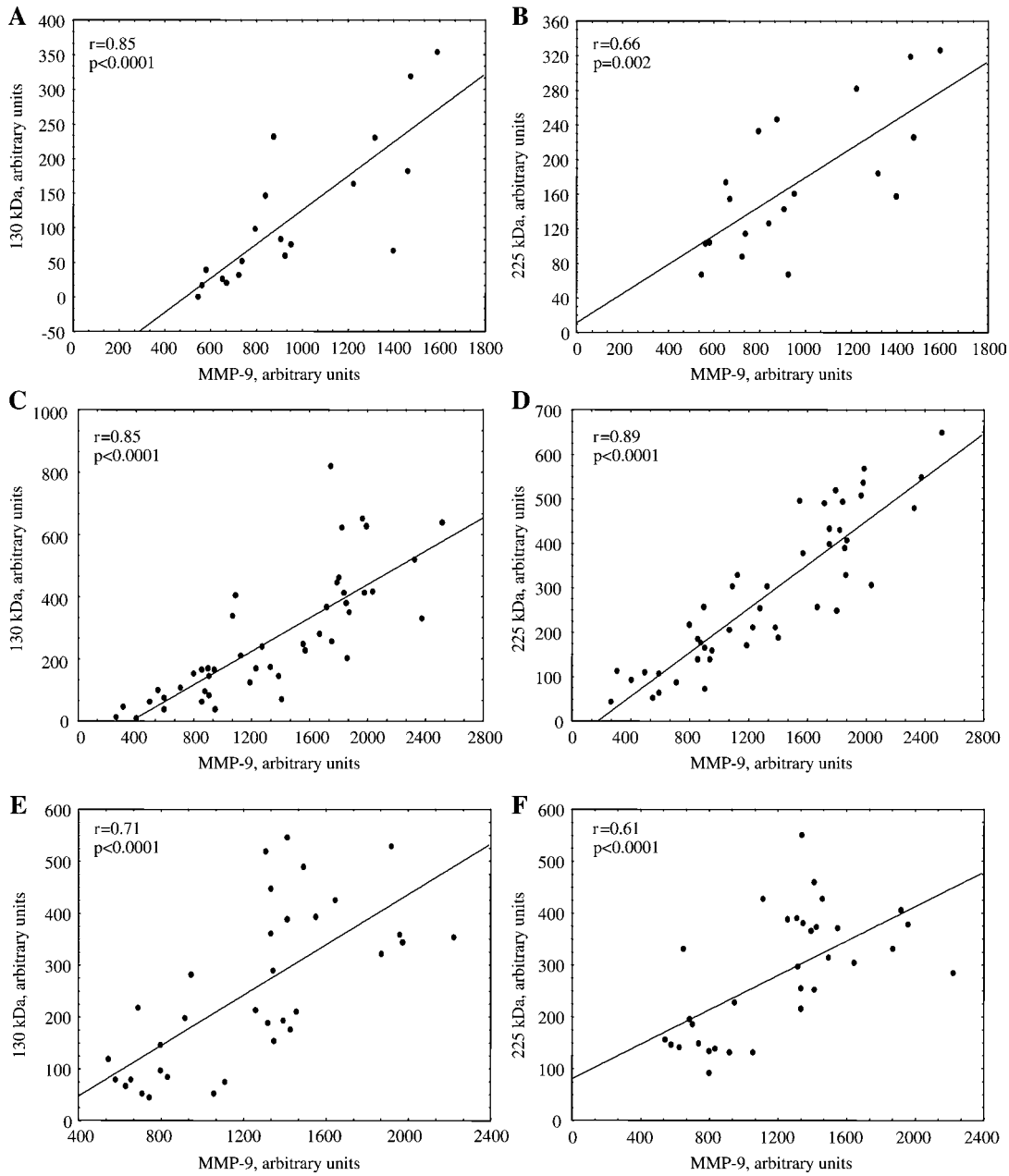

Figure 4 Correlation between MMP-9 levels and 130 kDa or 225 kDa gelatinolytic band values in the sera of normal controls (A and B, n = 19), and patients with colorectal (C and D, n = 47) or gastric (E and F, n = 34) cancer.

(24). Experimental data suggest that increased lipocalin-2 expression results in either significant stimulation of tumor growth (26) or lipocalin-2 may be a suppressor of colon cancer metastasis (27). However, we did not find any significant relationship between serum 130-kDa and histological grade and clinical stage in patients with colorectal or gastric cancer.

Our results showed that serum levels of MMP-2 in patients with colorectal or gastric cancer were modestly higher than normal controls, which is consistent with other observations using the same technique for measurement in serum (28). Compared to other reports where ELISA was used to determine MMP-2 in serum or plasma, there are conflicting results (3–5, 9, 10, 14, 29). We therefore suggest that zymographic determination of MMP-2 is of limited value for tumor grading, staging, and prognosis in either colorectal or gastric cancer (5, 10).

In the present study, significant increases in serum concentrations of CEA and CA 19-9 in patients with colorectal and gastric cancers could provide powerful and useful information regarding the progression of these specific types of cancer, consistent with other reports (30–33). Although the serum levels of MMP-9, 130- and 225-kDa gelatinolytic bands showed statistical significance similar to that exhibited by conventional tumor markers such as CEA and CA 19-9, none of them significantly correlate with CEA or CA 19-9 expression in serum. This may indicate that these are regulated independently from these tumor markers (9). Interestingly, the increased serum levels of gelatinolytic proteases could be related to neutrophil activation by some forms of gastrointestinal can-

cers. Furthermore, MMP-9 is reportedly expressed in normal cells, inflammatory conditions as well as in tumors from diverse sites, including skin, lung, breast, colo-rectum, liver, prostate, brain, bone marrow, and bone (34).

Gelatin zymography offers several features that render this technique particularly useful: several proteases (latent and active forms) of different molecular weights can be detected and evaluated with a single gel, a small amount of serum (0.2 μL) is used, and picogram quantities can be quantified. In addition, positive controls for both MMP-2 and -9 can be analyzed on each gel to alleviate inter- and intra-gel variation. However, the zymographic technique is difficult to scale up for routine use. Nonetheless, these features compare favorably with other methods, including ELISAs and Western blots, without the need for specialized and expensive materials such as antibodies (21, 35). On the other hand, discrepancies in results obtained by ELISA in colorectal or gastric cancer may be due to differences in monoclonal antibodies that recognize various forms of MMPs or their complexes with natural inhibitors TIMPs or α-macroglobulin. Gelatin zymography measures the total amount of MMPs by its proteolytic activity on gelatin substrate, because SDS dissociates enzyme-inhibitor complexes (36).

In conclusion, serum concentrations of MMP-2 were not significantly increased in patients with colorectal and gastric cancer. This may limit its value in these types of cancer. On the other hand, MMP-9, 130-kDa and 225-kDa gelatinolytic bands did significantly increase in patients with either colorectal or gastric cancer, and this increase is related to increases in proteolytic degradation of the extracellular matrix and matrix invasion (37). However, none of these significantly correlate with histological grade or clinical stage; thus we do not believe that their increase in serum is related to the extent of invasion and tumor progression. This may warrant measurement of these parameters in parallel with other conventional markers in a study of patients with these gastrointestinal cancers. Further studies are required to evaluate the benefits of determination of these parameters as prognostic or predictive biomarkers for screening, diagnosis and follow-up patients with colorectal or gastric cancer. This may improve diagnostic and therapeutic strategies.

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